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Dried alginate-entrapped enzymes (DALGEEs) and their application to the production of fructooligosaccharides

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ABSTRACT

A modification of the classical calcium alginate enzyme entrapment technique is described aiming to overcome some of the limitations of the former gel-based biocatalysts. DALGEEs (Dried ALGinate Entrapped Enzymes) were obtained dehydrating calcium alginate gel beads containing entrapped enzymes. A fructosyltransferase from *Aspergillus aculeatus*, present in Pectinex Ultra SP-L, was entrapped using this technique. The resulting DALGEEs were successfully tested both operating batchwise and in a continuous fixed-bed reactor for fructooligosacharide (FOS) synthesis from sucrose. Interestingly, DALGEEs did not re-swell upon incubation in concentrated (600 g/L) sucrose solutions, probably due to the lowered water activity (a_w) of such media. Confocal laser scanning microscopy of DALGEEs revealed that the enzyme molecules accumulated preferably in the shell of the particles. DALGEEs showed an approximately 30-fold higher volumetric activity (300 U/mL) compared with the calcium alginate gel beads. Moreover, a significant enhancement (40-fold) of the space-time-yield of fixed-bed bioreactors was observed when using DALGEEs as biocatalyst compared with gel beads (4030 g/day·L of FOS *vs.* 103 g/day·L). The operational stability of fixed-bed reactors packed with DALGEEs was extraordinary, providing a nearly constant FOS composition of the outlet during at least 700 h. It was also noticeable their resistance against microbial attack, even after long periods of storage at room temperature. The DALGEEs immobilization strategy may also be

useful for other biotransformations, in particular when they take place in low a_w media.

Keywords: enzyme immobilisation; entrapment; hydrogels; glycosidases; fructooligosaccharides; food oligosaccharides.

1. INTRODUCTION

Compared with covalent immobilization methods, enzyme entrapment in alginate beads is a low-cost approach that does not exert any chemical modification to the protein structure. Alginate is a highly negatively charged polysaccharide extracted from brown algae that forms rigid gel-like structures in presence of divalent cations. Enzyme entrapment in alginate is straightforwardly accomplished under mild conditions, providing outstanding activity recoveries and easy diffusion of substrates and products through the matrix pores [1-3]. Typically, alginate entrapment is widely applied to the immobilisation of living cells [4;5].

Alginate gel-based biocatalysts present several practical limitations such as a low volumetric activity and microbial contamination of the beads, even in the refrigerator, due to their high content of water and carbon [6;7]. Undesired enzyme leakage from the gel beads due to the size of the pores [6] is another typical disadvantage of alginate entrapment method. The diffusion out of the beads can be minimised by increasing alginate concentration and/or mannuronic (M)/guluronic (G) acid ratio, as well as decreasing the pH [8]. Among other strategies to avoid protein leakage, enzymes can be cross-linked with glutaraldehyde [9] or bound to a carrier such as activated coal [10] prior to the entrapment process. In another recent twist, the crosslinking of alginate with glutaraldehyde has been also explored [11;12]. Nevertheless, the complete removal

of toxic crosslinking chemicals is difficult and thus may be inappropriate for its application in food processing industries [13].

In this work, we have tackled some of the hurdles of calcium alginate immobilization method by applying a drying process to the gel beads. It is well reported that drying of alginate beads is widely applied in the delivery of drugs and enzymes (esp. in the detergents industry), since the beads, in contact with water, swell to their original size releasing the bioactive molecules [14;15]. Our hypothesis was that dried-alginate beads should not re-swell in a medium with low water activity (a_w) such as highly concentrated sugar solutions. In addition, the strategy of drying the calcium alginate gel-based biocatalyst could lead to an increased volumetric activity and thus a lower reactor volume to obtain a given productivity.

For this purpose, we selected the fructosyltransferase from *Aspergillus aculeatus* [16] and the resulting immobilized biocatalyst was characterized and tested in batch and continuous reactors for the production of fructooligosaccharides (FOS). FOS are fructose oligomers with a terminal glucose group in which 2-4 fructofuranosyl moieties are linked by different bonds (Fig. 1) [17-19]. Commercial FOS are mainly composed of 1-kestose, nystose and ^{1}F-fructofuranosylnystose [20]. They are produced at multi-ton scale from concentrated sucrose solutions using fungal transfructosylating enzymes such as those from *Aspergillus niger*, *Aspergillus oryzae* and *Aureobasidium pullulans* [21;22].

FOS are non-cariogenic ingredients, have a sweetness of 40-60% relative to sucrose, and exhibit prebiotic properties [23;24].

An effective immobilisation method of fructosyltransferases is highly desired as it would allow a continuous FOS production process, the reuse of the biocatalyst and the reduction of the total costs for industrial settings [25-27]. In addition, immobilisation often protects enzymes from inactivation at extreme pH, high temperatures or organic solvents [28;29]. In this work, the dried alginate biocatalysts were studied characterised, and their advantages over the classical alginate gel beads (volumetric activity, stability and space-time-yield) were highlighted.

2. MATERIALS AND METHODS

2.1. Materials.

Pectinex Ultra SP-L (batch no. KRN05409), a preparation from *Aspergillus aculeatus* used in fruit juice processing that contains pectinolytic, cellulolytic and fructosyltransferase activities [16], was kindly donated by Novozymes A/S (Denmark). Sodium alginate SG300 was obtained from Degussa Texturant Systems, Spain. Sodium alginate Algogel 6021 and Algogel 3021 were kindly donated by Cargill Iberica, Spain. Sucrose, glucose and fructose were from Merck. 1-Kestose and nystose were from TCI Europe. 1^F-Fructosyl-nystose was from Megazyme (Ireland). All other reagents were of the highest available purity.

2.2. Preparation and characterisation of dried alginate-entrapped enzymes (DALGEEs).

The gel beads were prepared by ionotropic gelation as described elsewhere [1] with some variations. A 4% (w/v) sodium alginate solution was prepared in distilled water and stirred until a homogeneous clear solution was observed. The solution was let to settle for 2 hours in order to eliminate all air bubbles. The alginate solution was then gently mixed in a ratio 1:1 (w:w) with the enzyme solution (Pectinex Ultra SP-L diluted 1:2 v/v with 10 mM sodium acetate buffer, pH 5.6). The resulting enzyme-alginate mixture was dropped with the help of a peristaltic pump (P-1, GE Healthcare) onto a 0.2 M $CaCl_2$ solution in sodium acetate buffer (pH 5.6, 10 mM). A pipette tip was used at the outlet of the pump and placed 5 cm over the $CaCl_2$ solution. The drops instantly formed gel beads in

contact with the $CaCl_2$ solution, which was maintained under magnetic stirring at 100 rpm. The beads were hardened in the $CaCl_2$ solution for 20 min with mild agitation. The diameter of the beads obtained was around 3 mm. The gel beads were then separated from the solution and washed twice with 250 mL of sodium acetate (pH 5.6, 10 mM) for 15 min under mild agitation. The gel beads obtained as described above were dehydrated by air flow or controlled evaporation at 35ºC until the size of the particles remained constant. The resulting beads were called DALGEEs (Dried ALGinate Entrapped Enzymes).

2.2.1. Confocal microscopy.

Protein distribution in DALGEE particles was studied using confocal fluorescence microscopy. Before immobilisation, proteins present in Pectinex-Ultra SP-L were labelled with fluorescein isothiocyanate (FITC), a fluorophore that chemically attaches to amino groups in proteins [30]. Pectinex Ultra SP-L was diluted 1:2 (v/v) in 0.1 M Na_2CO_3. Then, FITC dissolved in N,N-dimethylformamide was added to the protein solution in a ratio of 5 µg of FITC per milligram of protein [31]. The unbound FITC was removed using a pre-packed PD-10 column (Amersham Biosciences). The FITC-labelled protein sample was diluted 1:2, 1:100 and 1:1000 (v/v) with 10 mM acetate buffer (pH 5.6) before the immobilisation in calcium alginate and further drying. The resultant DALGEEs were analysed with a confocal laser scanning microscope (Leica, model TCS SP2-AOBS). An oil immersion objective (40.0 x 1.25) was used for all measurements and the pinhole aperture was set to 1.50 Airy (122 µm). The excitation wavelength was 488 nm and

the emission was collected at 500-550 nm, with the corresponding filters. Micrographs were taken at different plane depths. The exposure of the micrographs was adjusted using the software for image processing.

2.2.2 Porosimetry.

The specific surface area (S_{BET}) of the DALGEEs was determined from analysis of nitrogen adsorption isotherms at −196ºC using a Micromeritics ASAP 2010 equipment. The samples were previously degassed at 100ºC for 12 h to a residual vacuum of 5×10^{-3} Torr in order to remove any loosely held adsorbed species.

2.2.3. Water content.

A volumetric Karl–Fisher titrator (Mettler Toledo, model DL31) was used for measuring the water content of DALGEEs, employing Hydranal composite 5 as reagent.

2.2.4. Scanning electron microscopy

The DALGEEs were mounted on aluminium SEM stubs and sputter-coated with a thin layer of gold at completed Torr vacuum. Samples were examined by scanning electron microscopy using an XL3 microscope (Philips) at an acceleration potential of 20 kV.

2.3. Activity assays

The enzymatic activity towards sucrose was determined at 60ºC in 0.2 M sodium acetate buffer (pH 5.6) by measuring the release of reducing sugars from 100 g/L sucrose solution using the dinitrosalicylic acid (DNS) method [32]. The assay was adapted to 96-well microplates as described in our previous work [25]. One unit (*U*) of activity was defined as that catalyzing the formation of 1 μmol of reducing sugars per minute under the above conditions. The activity of the immobilised biocatalysts was determined incubating in an Eppendorf tube approx. 50 mg of alginate beads with 0.45 mL of 100 g/L sucrose solution in 0.2 M sodium acetate buffer (pH 5.6). The mixture was maintained at 60ºC and 600 rpm for 20 min in a Vortemp 56 incubator (Labnet). A 200 μL sample of the supernatant was withdrawn and submerged for 10 min in a water bath at 95ºC, in order to inactivate the possible lixiviated enzyme. Then, 50 μL of this sample were transferred to one of the wells of the microplate, and the concentration of reducing sugars was measured as described above.

2.4. Operational stability of DALGEEs in batch reactor

DALGEEs (approx. 10 mg) were incubated with 250 μL of 600 g/L sucrose solution at 35ºC and 900 rpm in a Vortemp 56 incubator (Labnet). After 20 minutes, the DALGEEs were separated from the reaction medium. The supernatant was incubated at 95ºC for 10 min in a water bath (in order to inactivate any possible lixiviated enzyme) and the content of reducing sugars was determined using the DNS method. The DALGEEs were washed twice with a cooled (4ºC) sucrose solution using high vortex agitation for 30 seconds each wash. A total of 13

reaction cycles, with washes in between, were performed to evaluate the operational stability of DALGEEs.

2.5. Operational stability of DALGEEs in a continuous fixed-bed reactor

DALGEEs containing the entrapped fructosyltransferase were packed in a HiTrap™ column with 1 mL of total volume (7 x 25 mm, GE Healthcare). The inlet of the column was connected to an isocratic pump with dual reciprocating pistons (model 515, Waters), in order to precisely control the flow of the feeding solution to the bioreactor (600 g/L sucrose in 0.2 M sodium acetate buffer, pH 5.6). Both the bioreactor and the feeding solution were maintained at 35ºC in an Ovan chamber incubator (Lovango). At different times, samples were taken at the outlet stream of the bioreactor and analysed by HPLC. When using gel beads, a column XK 16/20 (GE Healthcare) with 30 mL of total volume was used.

2.6. HPLC analysis

The analysis and quantification of the different carbohydrates present in the transfructosylation reactions was carried out by HPLC with a quaternary pump (Delta 600, Waters) coupled to a 4.6 x 250 mm Luna-NH_2 column (5 µm, 100 Å) from Phenomenex. Detection was performed using an evaporative light scattering detector DDL-31 (Eurosep) equilibrated at 85ºC. Acetonitrile/water 75:25 (v/v), degassed with helium, was used as mobile phase at 0.9 mL/min for 3 min. Then, a gradient from this eluent to acetonitrile/water 70:30 (v/v) was performed in 1 min, and held for 2 min. A new gradient to acetonitrile/water 60:40 (v/v) was

performed in 2 min and held for 6 min. Total analysis time was 14 min. The column temperature was kept constant at 25°C. The data obtained were analysed using the Millennium Software.

2.7. Water activity

Water activity of sugar solutions was determined using a humidity and temperature digital indicator Thermoconstanter TH200 (Novasina, Switzerland). The humidity sensor was calibrated with control saturated salts solutions of different a_w values (LiCl, 0.11; potassium acetate, 0.22; NaBr, 0.57; NaCl, 0.75; $K_2Cr_2O_7$, 0.98) at 25°C.

3. RESULTS AND DISCUSSION

3.1. Preparation and characterisation of dried alginate-entrapped enzymes (DALGEEs)

Fructosyltransferase activity from *A. aculeatus,* present in Pectinex Ultra SP-L [16;33], was entrapped in calcium alginate beads using different commercial sodium alginates (SG300, Algogel 6021 and Algogel 3021) and their immobilisation yields were compared (Table 1). The immobilized enzymatic activity of the alginate beads was determined indirectly by measuring the initial activity of the solution before gelation and the activity present in the $CaCl_2$ and washing solutions. The value calculated was considered as the theoretical immobilised activity. The main contribution to the loss of activity occurs while the drop is not fully gelled to form the bead [34]. Table 1 shows that sodium alginate SG300 was the most adequate for our purpose, probably due to an optimal mannuronic/guluronic acid ratio, with an immobilisation yield close to 50%. This value was also in accordance with the total protein immobilisation yield (47%) measured by the Bradford assay. Initial protein concentration in Pectinex preparation was approx. 17 mg/ml and fructosyltransferase accounted for only 0.4% of total protein [16]. Theoretical immobilisation yields in the range 40-80% are usually reported [1;9;11;13] and, therefore, the SG300 alginate was selected for further experiments.

The so-called apparent activity (experimental) of the immobilised biocatalysts, which takes into account mass transfer and diffusional restrictions

[35], was measured using 3 gel-beads (approx. 50 mg) and 100 g/L sucrose. The volumetric apparent activity of the SG300 gel-based biocatalyst was 10 U/mL, which is nearly 4-fold lower than the theoretical value. The difference between theoretical and apparent activities seems to be related with the mass transfer of substrates and products within the alginate matrix.

We performed the drying process of the calcium alginate gel beads and the resulting biocatalysts, with a crystalline appearance, were called DALGEEs (Dried ALGinate Entrapped Enzymes). The size of the alginate beads was significantly reduced, from 3 mm to less than 1 mm upon drying, which accounted for a volume reduction of approx. 96% (considering the beads as perfect spheres). The images obtained by scanning electron microscopy of the DALGEE particles are shown in Fig. 2. Beads were nearly spherical and quite homogeneous in size, showing a rough surface. Santagapita et al. reported that roughness or smoothness is affected both by the beads composition and drying method [36].

A crucial point for the applicability of DALGEEs was to analyse if they rehydrated when they were submerged in the sugar solution, as swelling may be accompanied by enzyme leakage. Fig. 3 shows that DALGEEs rehydrate in buffer solution. In contrast, DALGEEs beads maintain its initial size after 1 day incubation in 600 g/L sucrose. This fact seems to be related with the decrease of water activity (a_w) caused by sucrose [37]. This property is important since many chemical, enzymatic and microbiological processes are dependent on the availability of water [38;39]. The DALGEEs swell very slightly when they are in

contact with a concentrated sucrose solution; this smooth swelling may facilitate the contact between the enzyme molecules and the substrates. We observed that a 600 g/L sucrose solution lowers the a_w of water from 1.0 to 0.97; this small change is enough to prevent DALGEEs from recovering their initial size. When increasing sucrose concentration up to 950 g/L, the a_w diminishes to a value of 0.87. However, the swelling process may be also influenced by the M/G ratio of the alginate and/or the drying method.

The apparent enzymatic activity of DALGEEs containing fructosyltransferase from *A. aculeatus* was 300 U/mL, which is 30-fold higher than that measured for the gel beads. The textural studies revealed the low porosity of this material, with a BET area of 7.6 m^2/g and a total pore volume of 0.072 cm^3/g, measured from the adsorption nitrogen isotherms. The water content of DALGEEs, determined by the Karl Fisher assay, was of only 1.5 % (w/w).

The distribution of immobilised FITC-labelled proteins in DALGEEs was studied by fluorescence confocal microscopy. Although fluorescence was found throughout the whole volume of the beads, a clear preferential accumulation of the enzyme in the shell of the particle was observed when lowering the exposure of the emission signal (Fig 4A). This result is in accordance with the expected hypothetical drying process, as it occurs from the outer to the inner layers thus enriching the beads with protein in the external layers (Fig. 4B) [40].

3.2. FOS synthesis in batch reactor with fructosyltransferase in DALGEEs

The reuse of DALGEEs was studied in a batch reactor measuring the activity of the beads in a sequence of reaction cycles. Fig. 5 illustrates the operational stability of the DALGEEs in 13 successive reaction cycles of 20 min each. As it is shown, the operational stability of DALGEE biocatalysts was very satisfactory. This behaviour was probably related with the fact that DALGEEs do not swell in concentrated sucrose solution and thus the enzyme leakage is minimised.

3.3. FOS synthesis in continuous fixed-bed bioreactors with fructosyltransferase in DALGEEs

A fixed-bead reactor of small volume (1 mL) was packed with the DALGEEs. The operation of the continuous reactor for FOS synthesis was assayed at 0.01 mL/min and 35ºC. The bioreactor was operated continuously for 700 h during which samples were taken and analysed by HPLC. As shown in Fig. 6, the composition of the outlet was nearly constant at least during 700 h, with an average FOS concentration of 275 g/L, which indicated an optimal operational stability of the DALGEEs biocatalysts. The composition of the outlet was approximately as follows: 175 g/L glucose, 148 g/L sucrose, 140 g/L 1-kestose, 96 g/L nystose, 30 g/L 1^F-fructosyl-nystose and 11 g/L 1^F-fructosyl-fructosyl-nystose. Considering the volume of the bioreactor and the mean concentration of total FOS measured at the outlet, the space-time yield was approx. 4030 g FOS/ day · L.

The above space-time yield was compared with that of a 25 mL fixed-bed reactor packed with the calcium alginate gel beads, which was also operated in continuous mode, at 35ºC and with a flow rate of 0.01 mL/min. Samples at the outlet of the column were analysed by HPLC. The sucrose conversion reached with this system was 67%, and the space-time yield was 103 g FOS/ day · L, which is about 40-fold lower than that determined for a reactor packed with DALGEEs.

The fructooligosacharide composition obtained from enzymatic transfructosylation is highly dependent on the reaction time [25], which is proportional to the residence time in a continuous reactor. The FOS composition was therefore studied operating the reactor at different flow rates in order to control the residence time. The results of this study (Fig. 7) showed that high residence times led to a final FOS product enriched in tetra- and pentaoligosaccharides, whereas low residence times yielded to a trisaccharide-enriched FOS mixture. The specific effects of prebiotic oligosaccharides depend, among other factors, on their degree of polymerization [18;41;42], so it is interesting to control the FOS distribution varying the flow rate of the fixed-bed reactor. The study also revealed that an increase of the residence time was translated into higher sucrose conversion values, achieving a maximum value of 91%. Elsewhere, the extraordinary operational stability of DALGEEs biocatalysts in continuous fixed-bed reactors ensures a product with a constant FOS composition.

This immobilization method may also be suitable for other biotechnological processes, in particular those involving the transformation of carbohydrates.

Indeed, we have also applied the DALGEEs technology to obtain glucose/fructose syrups using a β-fructofuranosidase from *Rhodotorula dairensis* [43], to convert inulin into fermentable sugars using an inulinase, and for galactooligosaccharides synthesis using a β-galactosidase (data not shown).

CONCLUSIONS

The methodological simplicity for the preparation of DALGEEs and the low cost of the required materials make this process attractive for its application in large-scale FOS synthesis. The drying process of the gel beads resulted in particles with crystalline appearance and increased volumetric activity (30-fold) compared with the corresponding gel-based biocatalysts. In addition, its storage stability (absence of microbial growth compared with the hydrated beads), operational stability and no swelling when used in low a_w media (e.g. concentrated sucrose solutions) offer excellent perspectives for applications in biotransformations.

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Figure legends

Fig. 1. Molecular structure of fructooligosaccharides (FOS) synthesized in this work.

Fig. 2. SEM micrographs of the DALGEE particles. Magnifications: upper picture, 45x; bottom picture, 1000x.

Fig. 3. Swelling of DALGEEs obtained with Algogel 3021: (1) Alginate gel particle; (2) DALGEE particle; (3) DALGEE particle after 24 h incubation in buffer; (4) DALGEE particle after 24 h incubation in 600 g/L sucrose.

Fig. 4. (A) Confocal image of FITC-labeled proteins, from Pectinex Ultra SP-L, entrapped in DALGEE particles. The images belong to a DALGEE particle in which the FITC-labelled protein solution was diluted 1:100 (v/v) prior to the gel-entrapment procedure. (B) Proposed mechanism of protein concentration on the shell of a DALGEE particle upon the drying process.

Fig. 5. Reuse assay of DALGEEs, containing the fructosyltransferase from *A. aculeatus,* in successive batch reactions. After each reaction cycle (20 min), the DALGEEs were separated from the reaction medium and washed. Reaction conditions: 600 g/L sucrose, 35ºC, 900 rpm.

Fig. 6. Operational stability of the fixed-bed bioreactor packed with DALGEEs containing the fructosyltransferase from *A. aculeatus*. Reactor conditions: Feed, 600 g/L sucrose; Flow rate, 0.01 mL/min; Temperature, 35°C.

Fig. 7. Effect of the residence time on the sucrose conversion and FOS composition in the fixed-bed bioreactor packed with DALGEEs containing the fructosyltransferase from *A. aculeatus*. Operational conditions: Feed, 600 g/L sucrose; Temperature, 35°C.

Table 1. Effect of the source of alginate on the immobilisation by entrapment of *A. aculeatus* fructosyltransferase.

Alginate	Total activity before immobilisation (U)	Immobilised activity [a] (U)	Volume of gel beads [b] (mL)	Volumetric activity [c] (U/mL)	Immobilisation yield [c] (%)
SG300	274.0	138.8	3.4	40.7	50.7
Algogel 6021	193. 6	34.9	2.4	14.5	18.1
Algogel 3021	180.9	46.4	2.3	20.6	25.6

[a] Determined by subtracting the total initial activity in the solution prior to its gelation and the activity in the remaining $CaCl_2$ and washing solutions.

[b] The gel-based biocatalyst volume was estimated considering a gel density of 1 g/mL.

[c] Theoretical values.

Fig. 1

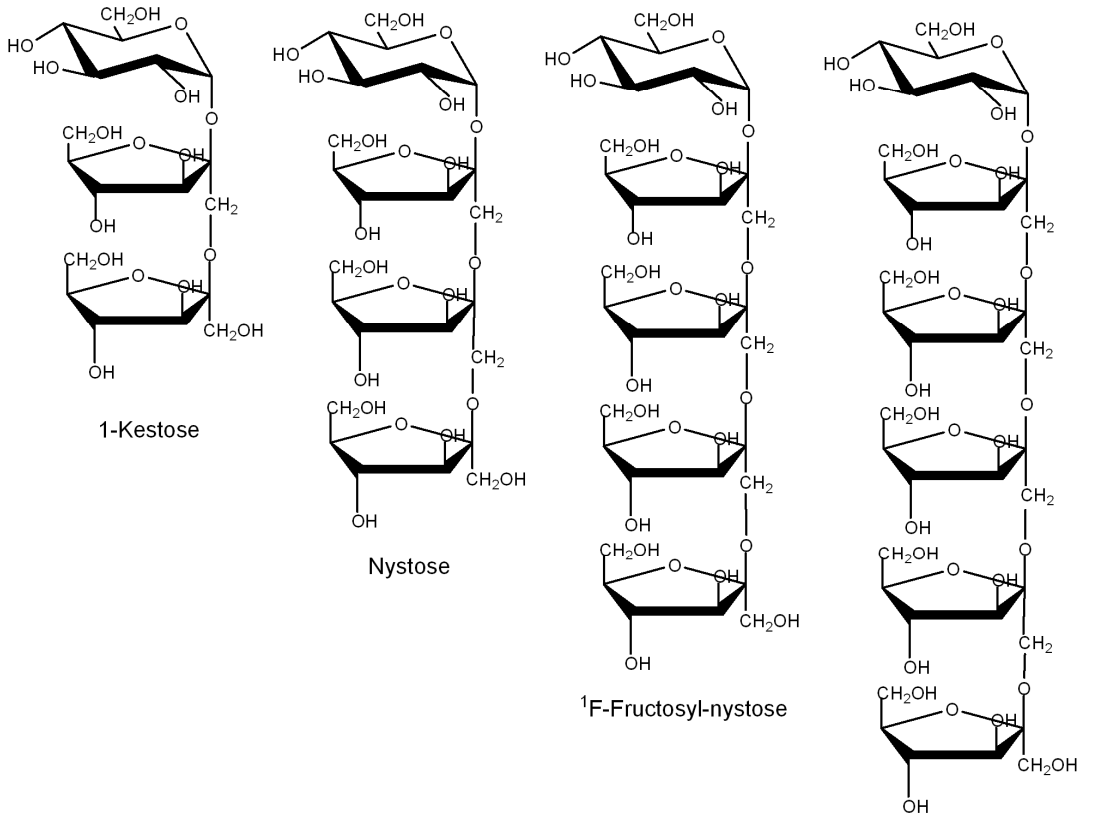
1-Kestose
Nystose
1F-Fructosyl-nystose
1F-Fructosyl-fructosyl-nystose

Fig. 2

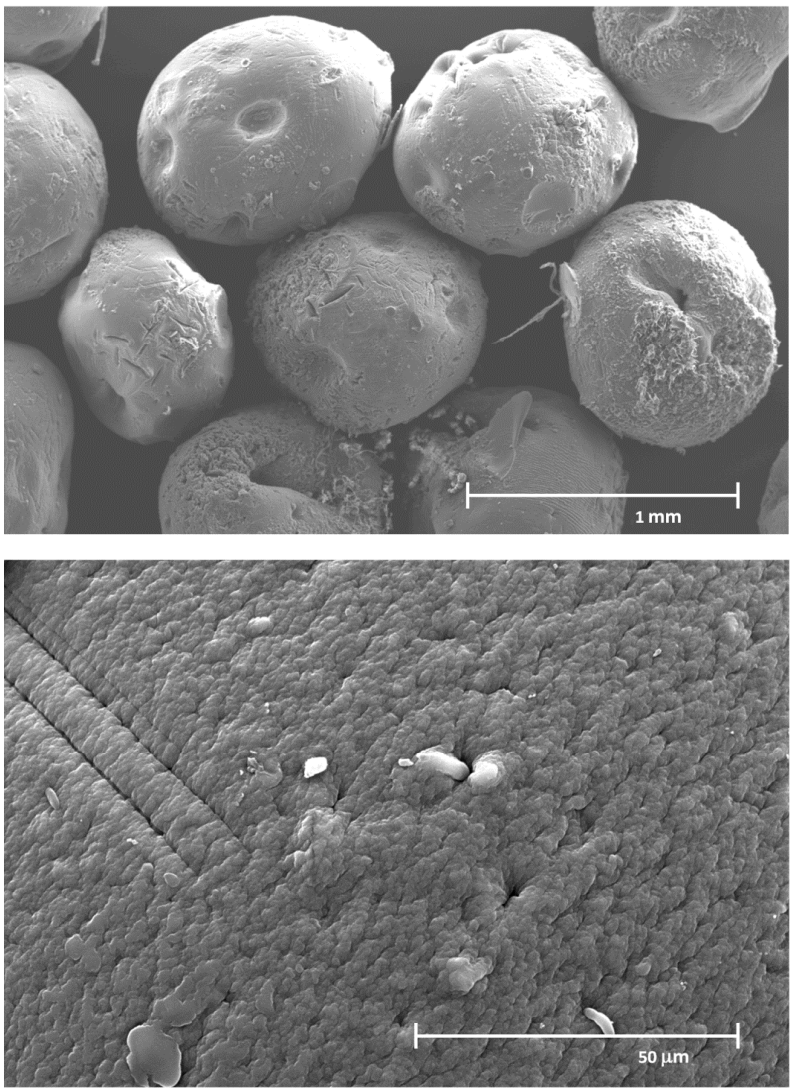

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Fig. 3

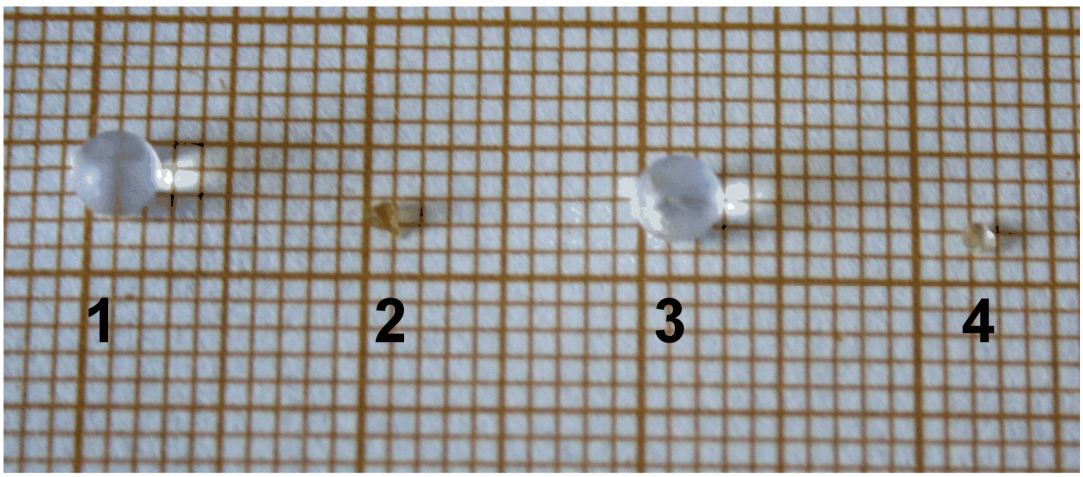

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Fig. 4

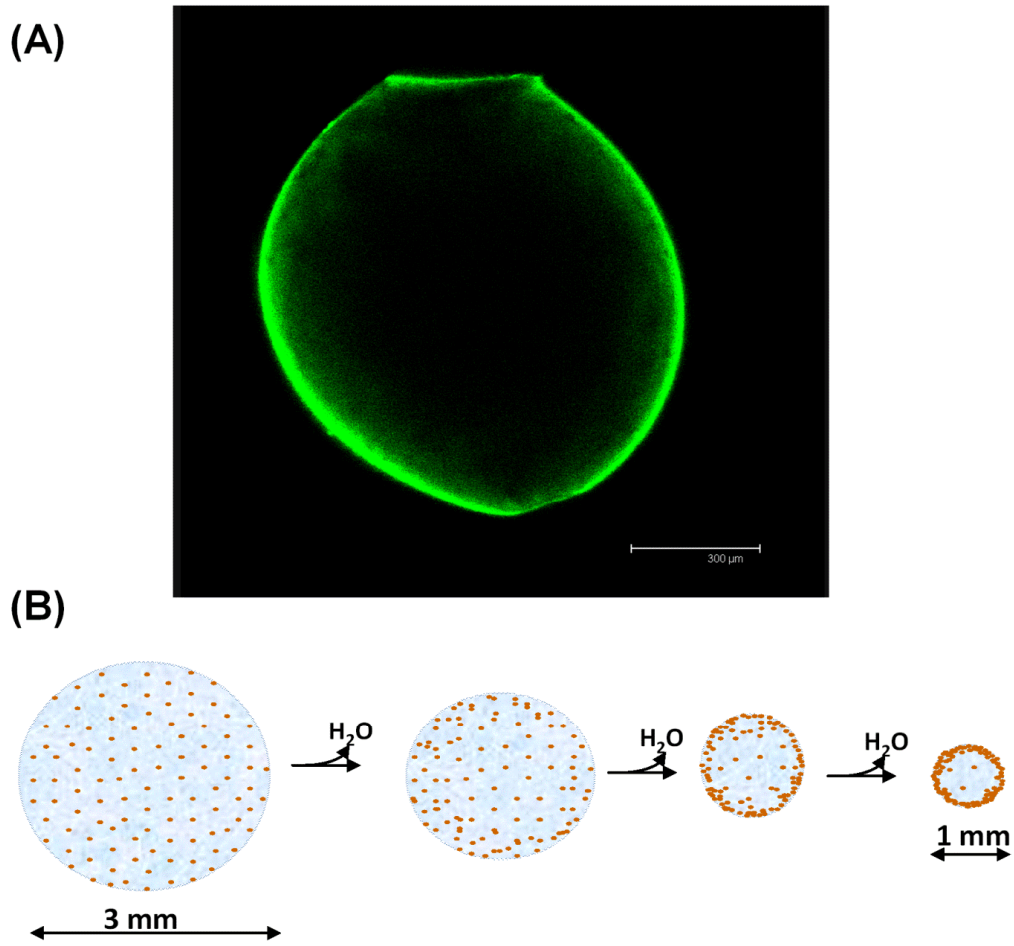

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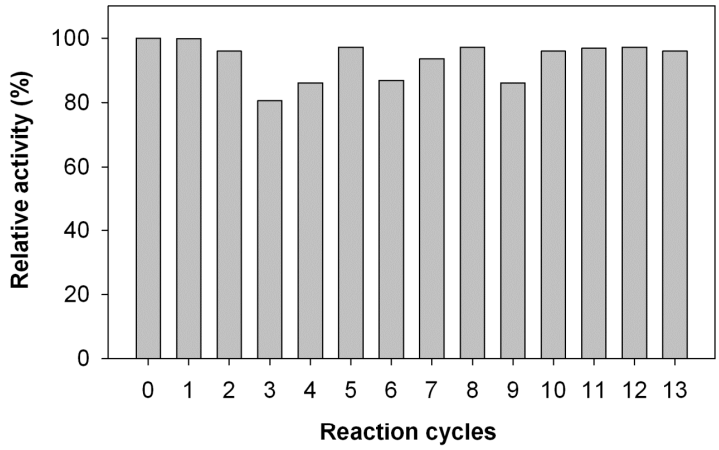
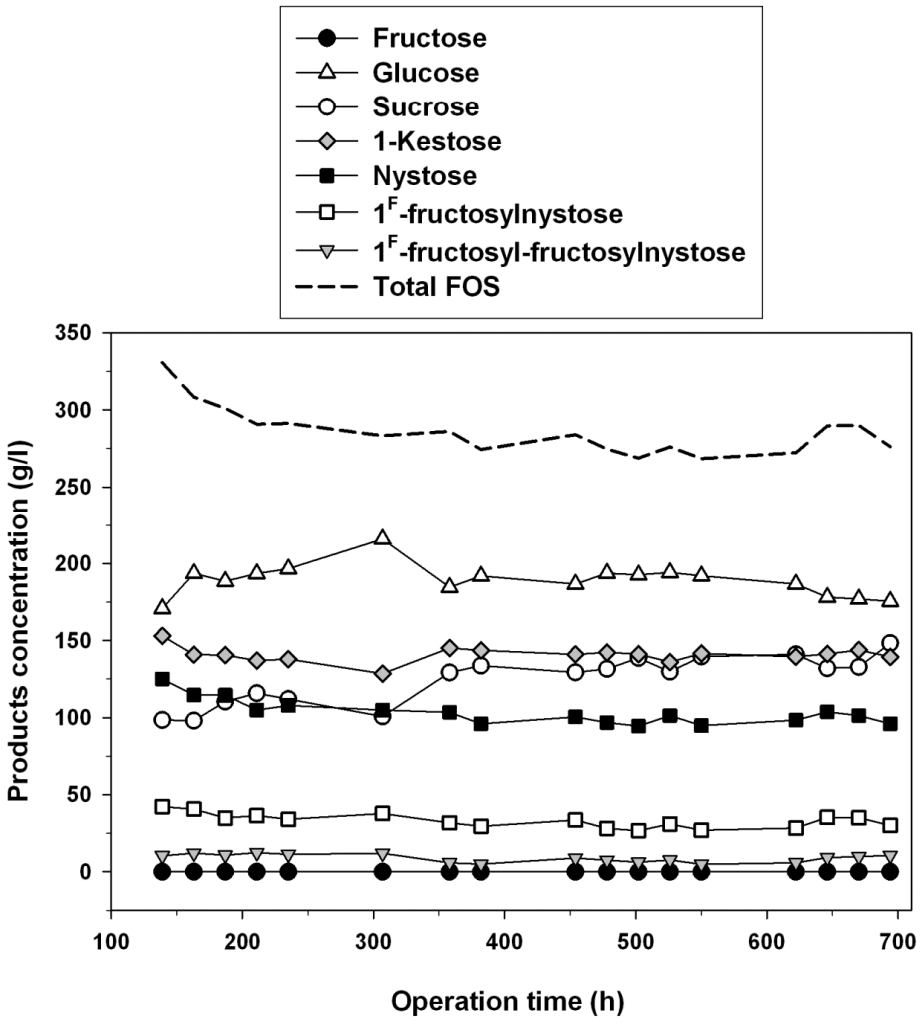
Fig. 5


Relative activity (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Reaction cycles

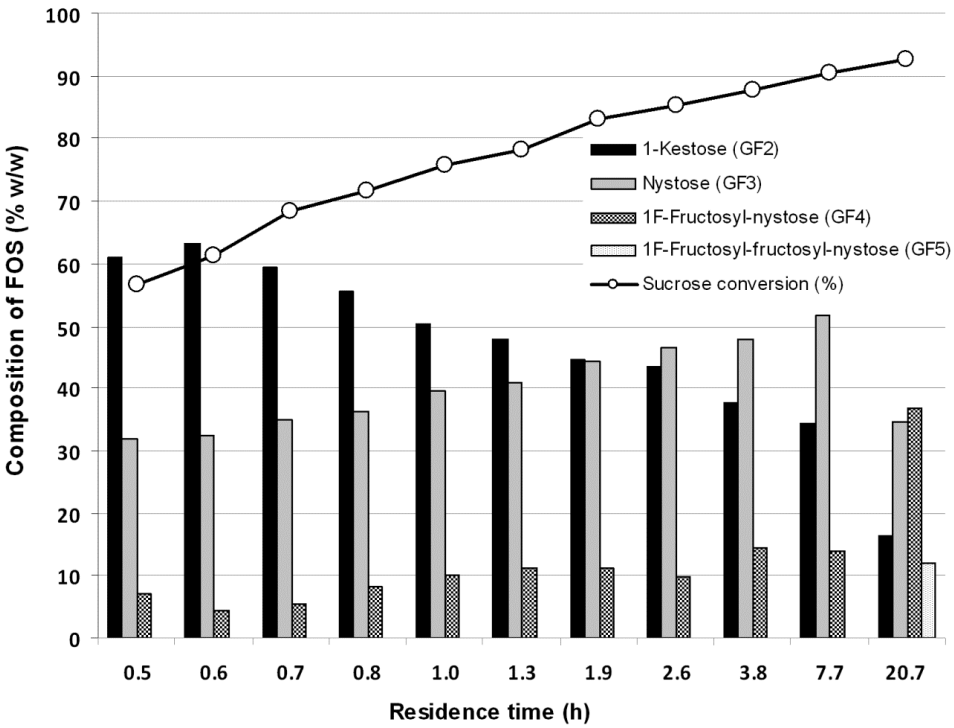
1

Fig. 6


Fructose
Glucose
Sucrose
1-Kestose
Nystose
1F-fructosylnystose
1F-fructosyl-fructosylnystose
Total FOS
Products concentration (g/l)
Operation time (h)
350
300
250
200
150
100
50
0
100
200
300
400
500
600
700

1

Fig. 7


Composition of FOS (% w/w)
100
90
80
70
60
50
40
30
20
10
0
0.5
0.6
0.7
0.8
1.0
1.3
1.9
2.6
3.8
7.7
20.7
Residence time (h)
1-Kestose (GF2)
Nystose (GF3)
1F-Fructosyl-nystose (GF4)
1F-Fructosyl-fructosyl-nystose (GF5)
Sucrose conversion (%)